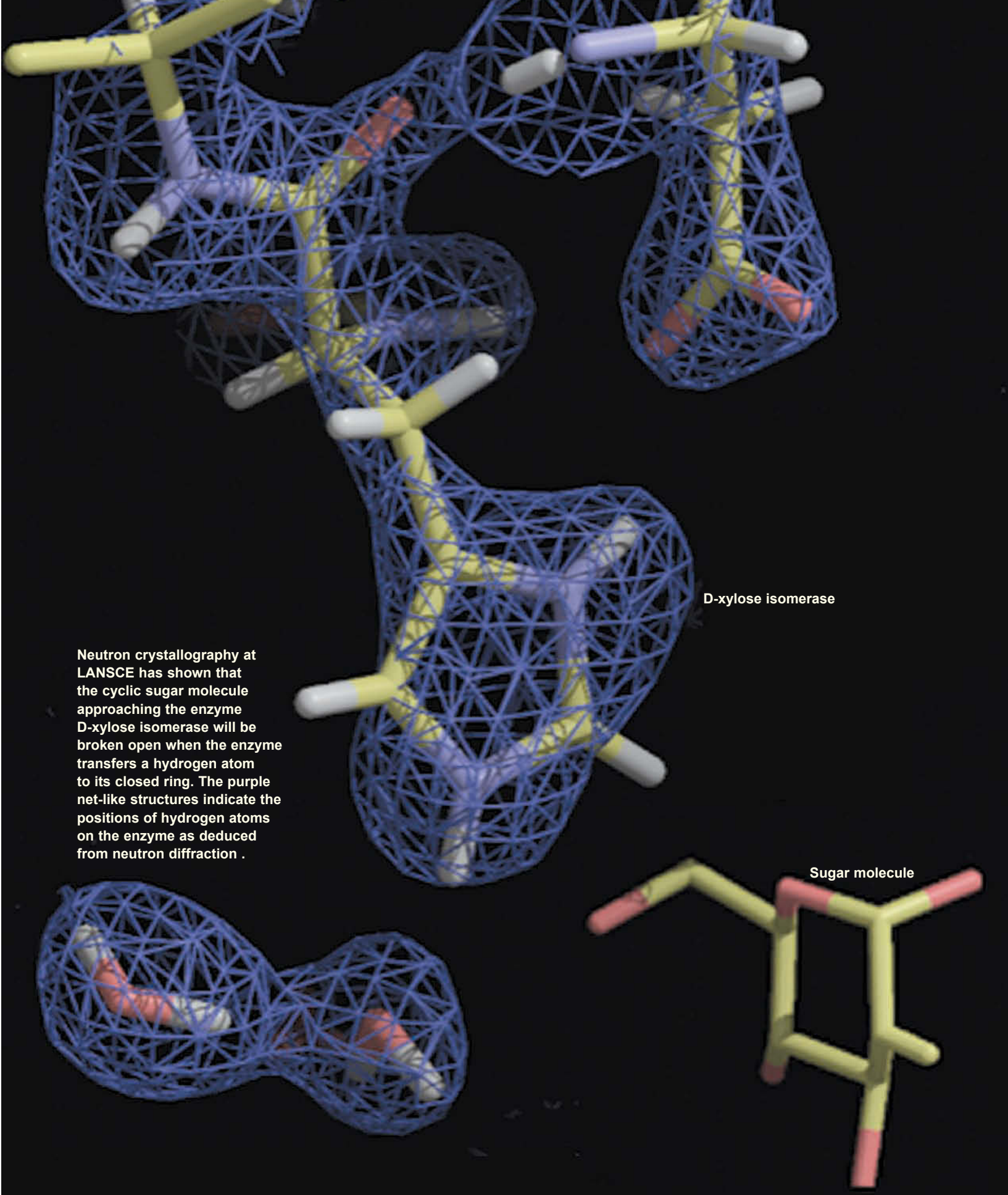
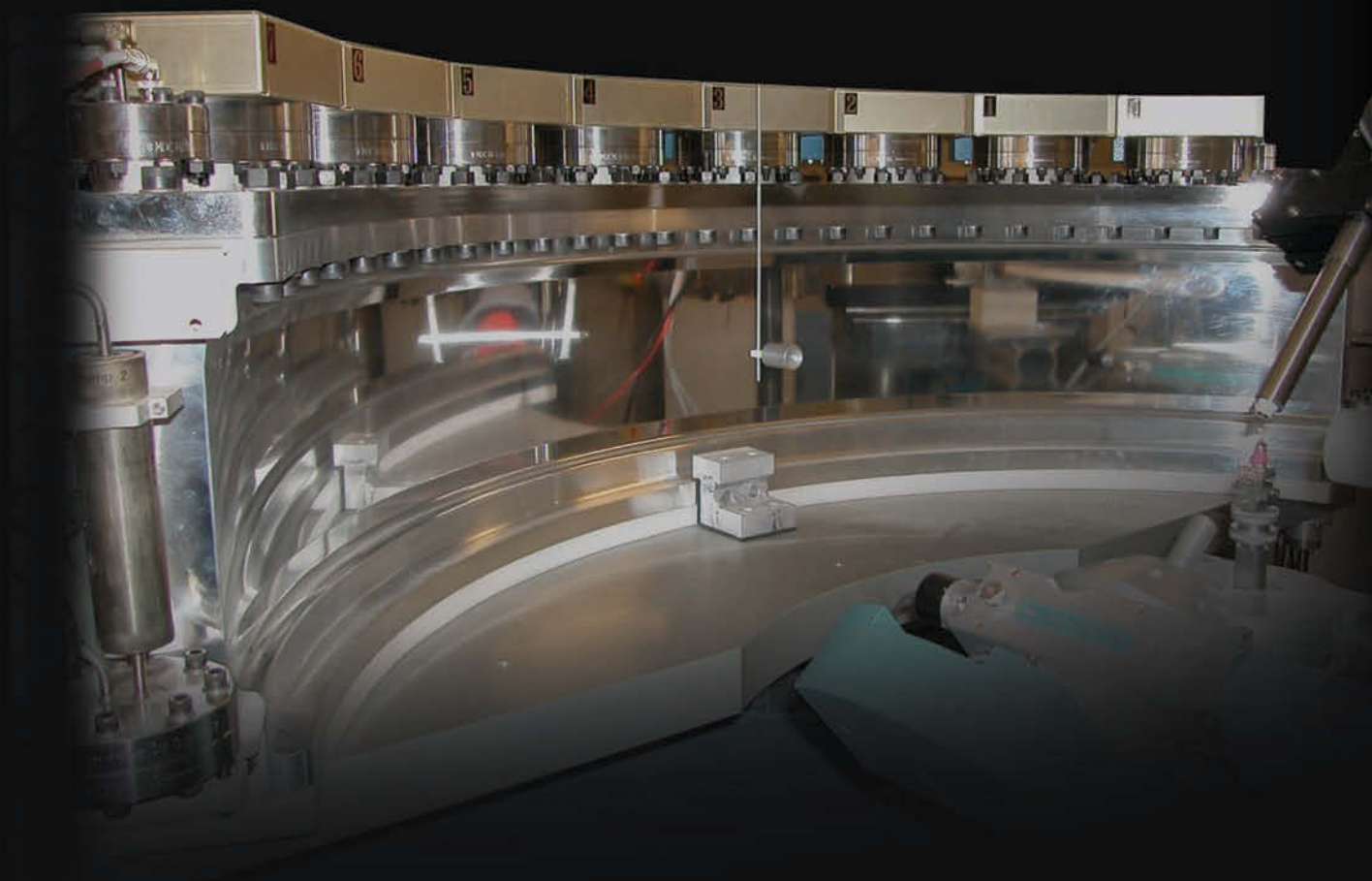


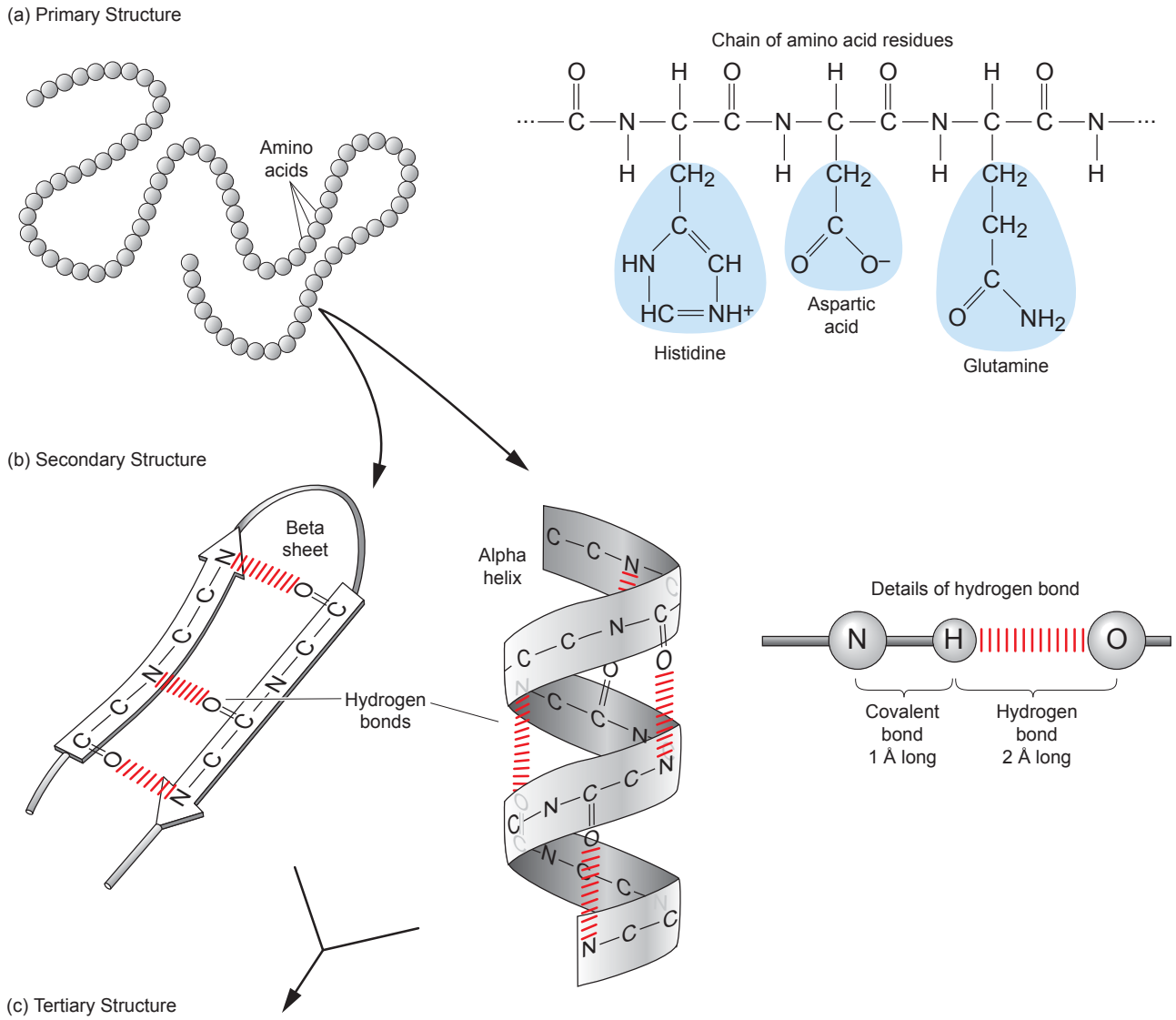
Neutron crystallography at LANSCE has shown that the cyclic sugar molecule approaching the enzyme D-xylose isomerase will be broken open when the enzyme transfers a hydrogen atom to its closed ring. The purple net-like structures indicate the positions of hydrogen atoms on the enzyme as deduced from neutron diffraction .

Finding out How Enzymes Work

Paul A. Langan

The new Protein Crystallography Station at LANSCE is making it possible to pinpoint the biochemical steps involved in enzyme reactions. That knowledge should improve our overall understanding of cellular processes and of how defective and pathogenic enzymes affect our health. It should also enable the design of more effective drugs and therapeutics for treating disease.





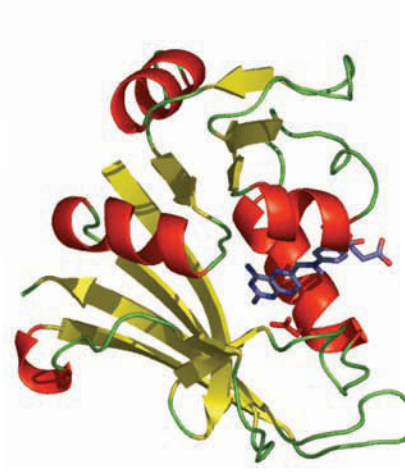


Figure 1. Protein Structure

Proteins are constructed from individual building blocks known as amino acids. The amino acids polymerize, or join together, to form long chains of amino acid residues. (a) Shown here are a cartoon of the primary chainlike structure of a protein and an expanded view, showing three amino acid residues—histidine, aspartate, and lysine—strung on the protein backbone. (b) The secondary structure is the folding of the chain into either (1) beta sheets, in which segments of the amino acid chain, represented by ribbonlike arrows, lie parallel or antiparallel to each other and are held in place by hydrogen bonds (red dashed lines) connecting the backbones of the segments or (2) alpha helices, a helical winding of the chain held in place by a scaffold of hydrogen bonds parallel to the axis of the helical winding and again connecting the backbones of parallel portions of the chain. (c) The tertiary structure is a single chain folded into alpha helices, beta sheets, and intervening loops to form a complicated structure with clefts, barrels, pockets, and cages.

Within each of the microscopic cells that make up our bodies, specialized protein molecules, called enzymes, are frantically working away shuffling atoms about in chemical reactions that make those cells come alive. Energy production, cell growth, cell division, and other essential life processes involve thousands of such reactions, many with their own specialized enzymes, interconnected through complex pathways and networks. Like all proteins, an enzyme is created by linking small molecules, called amino acids, into a long chain. About twenty kinds of amino acids, each with different chemical properties, are found in our cells. Many of these amino acids—for example, aspartic acid, glutamine, and lysine—are found in food supplements. Each type of enzyme is composed of a different sequence of tens, sometimes hundreds, of amino acids, and that sequence determines how its chain folds into a unique three-dimensional shape with features such as helices, barrels, sheets, loops, pockets, clefts, and cages (Figure 1). The shape of an enzyme is key to understanding how it works, and understanding how enzymes work is key to understanding cellular life processes.

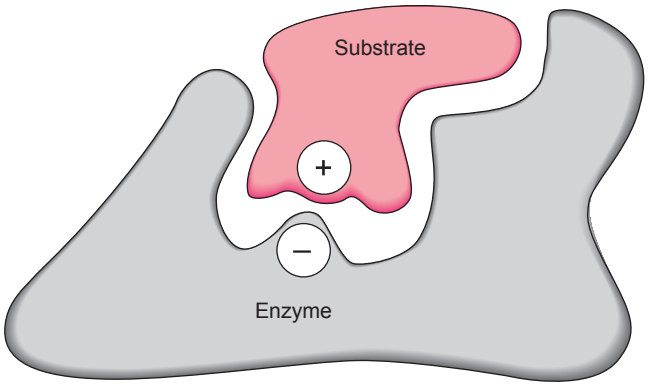


Figure 2. Enzyme-Substrate Binding
An enzyme begins its catalytic function by binding a particular substrate to its active site. The two fit together like a lock and key. In addition to shape, a unique pattern of attractive forces and hydrogen bonds makes the active site highly selective for one particular substrate.

The Structure-Function Relationship

The shape of a molecule, which scientists call its structure, can be revealed using a technique called x-ray crystallography, developed in the last century. Over the past 50 years or so, x-ray crystallography has been used to reveal the structure of a number of enzymes. These structures, together with information from their chemical analysis, have led to a clear set of ideas about how an enzyme might work. The essential job of an enzyme, speeding up a particular chemical reaction, can be thought of as involving two steps: binding a chemical that the enzyme will work on, called a substrate, and then pushing the chemical reaction to occur, called catalysis. Binding takes place at an active region of the surface of the enzyme where the enzyme and substrate have complementary shapes. The substrate fits into the active site like a key in a lock (Figure 2). However, the lock-and-key model is not enough to explain binding; attractive forces are needed to hold the substrate in place. These forces include electrostatic attractions between oppositely charged groups and also chemical bonds that are mediated by hydrogen atoms, called hydrogen bonds. The unique pattern of these interactions makes the active site of an enzyme highly selective for its particular substrate.

During catalysis, as the atoms in the enzyme and substrate begin to react with each other to weaken old bonds and to begin to form new bonds, the reacting atoms must overcome forces that tend to push them apart. Pushing atoms together into a momentary transition state through which the reaction must pass requires energy. This extra energy can be thought of as a hill or barrier that must be climbed before the reaction can take place. A key insight, made by the famous chemist Linus Pauling in the early years of studying enzymes, is that enzymes bind the transition state better than the substrate, and this process reduces the height of the energy hill. More recently, scientists have mapped out other possible routes for ascending the energy hill. One of these is based on the idea that binding a substrate on the surface of an enzyme reduces its entropy (the amount of freedom to move) and increases its free energy, and that this free energy can be used to climb the energy hill. It has even been proposed that small particles such as electrons and hydrogen atoms can tunnel through the energy hill rather than climb over it, by using quantum physical processes. These ideas on substrate binding and catalysis have led to a number of medical advances, particularly in the design of new drugs and therapeutics.

Protein Structure and Drug Design

At present, x-ray crystallographic studies of enzymes are mostly con-

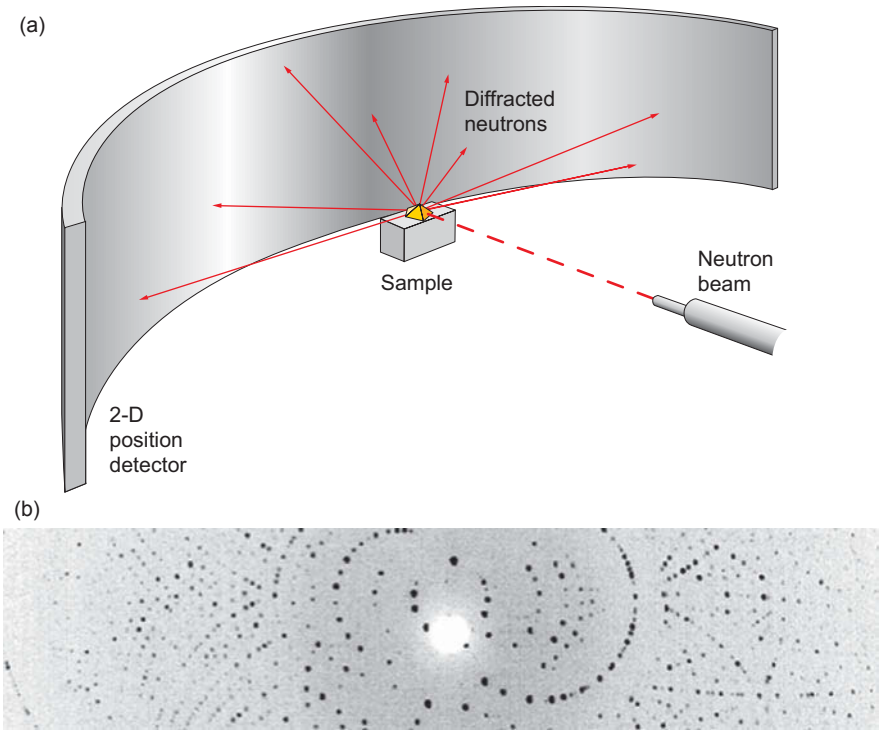


Figure 3. Diffraction
(a) In a neutron diffraction experiment at the LANSCE Protein Crystallography Station (PCS), a protein crystal is placed in a neutron beam, and the diffracted rays are recorded on a large two-dimensional electronic detector. (b) This diffraction pattern of the protein D-xylose isomerase taken at the PCS shows a regular array of diffraction peaks. These peaks are analyzed to reveal the location of hydrogen atoms in the protein structure.

ducted at large central synchrotron x-ray sources that are used by many groups of visiting scientists and that are powerful enough to visualize the structure of thousands of enzymes a year. As a result of the Human Genome Project and its spinoffs, the amino acid sequence of any enzyme that is encoded in a gene can now be read, and then that enzyme can be synthesized for study even if it has never been seen before and its function is completely unknown. There is great hope that, by revealing the structure of all proteins and enzymes, we will begin to understand how they work in detail and that this awareness will provide an overall understanding of cellular processes and of how defective or pathogenic enzymes can affect our health. Understanding how enzymes work in greater detail may also provide further clues about how they could be improved or modified for medical applications or for applications in industries such as food, agriculture, and remediation.

One of the most powerful benefits of our improved understanding of how enzymes work has been to the design of new drugs and therapeutics. Many diseases, such as cancer, involve enzymes that don't work properly or that are not produced or regulated properly. Drugs can bind to an enzyme and stop it from working in at least two ways: competitive inhibition and noncompetitive inhibition. In competitive inhibition, a drug competes with the substrate to bind at the active site of the enzyme. The goal is to design a drug that binds more strongly in the active site than the enzyme's natural substrate. A drug is designed to bind in a region next to the active site in noncompetitive inhibition. The drug prevents substrate binding by distorting the shape of the active site, so that the key no longer fits the lock. Structure-based drug design has already produced several life-saving drugs, such as Fortovase®, Norvir®, Crixivan®, Viracept®, Agenerase®, Trusopt®, Relenza®, Celebrex®, Vioxx®, and Thymitaq® that are vital for people with health problems such as AIDS, arthritis, glaucoma, flu, and cancer. Many more drugs are in the pipeline.

Deciphering Function with Neutrons

Despite these advances, a limitation in using x-rays to study enzymes is that they only reveal the enzyme's "skeleton." Although x-rays "see" the atoms of carbon, oxygen, sulfur, and nitrogen that connect up the skeleton of a protein, they have a much harder time seeing the smaller, more-mobile atoms of hydrogen that are attached to this skeleton and that make up the water molecules that surround and interact with the protein. The reason for this observation is that x-rays interact primarily with the electrons surrounding the nucleus of an atom; the more electrons an atom has, the stronger its interaction with x-rays. With only one electron, a hydrogen atom is usually invisible to x-rays. This limitation is severe because hydrogen atoms—whether they are involved in folding the enzyme or binding the substrate, whether they are shuffled about during catalysis or take part in interactions of the enzyme with its aqueous environment—are crucial to enzyme function. Without knowledge of where the hydrogen atoms are in an enzyme and how they are moved about during binding and catalysis, it is difficult to

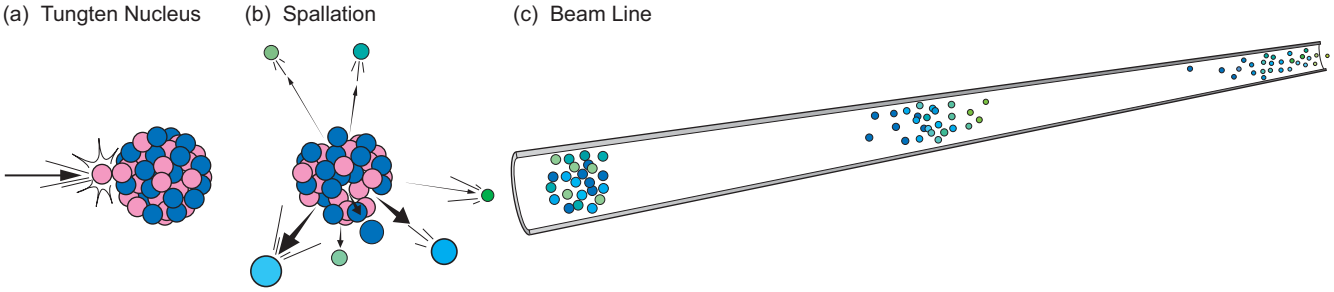


Figure 4. Spallation Neutrons
(a) When an energetic proton hits the nucleus of a heavy atom, (b) the nucleus releases tens of neutrons with different energies. (c) The resulting pulse of neutrons is very short, but as the pulse travels down the flight path toward the experiment, the higher-energy neutrons travel faster, the pulse stretches out in space, and the arrival times of the neutrons serve to identify their energies. This temporal separation of neutrons by energy allows collection of separate diffraction patterns, each corresponding to a different neutron energy (or wavelength).

know how an enzyme works in detail and how to design a drug that will bind more effectively.

In order to solve the problem of finding hydrogen atoms, neutron crystallography has been developed and applied to study enzymes over the past 40 years. Like x-ray crystallography, neutron crystallography can be used to reveal structure by a process called diffraction. A sample made from crystallized protein molecules is placed in a beam of neutrons, and the atoms scatter neutrons out of the beam. Because the protein molecules are packed in a very regular way in the crystal, neutrons are scattered strongly only in certain directions and make a pattern of spots, called a diffraction pattern, when they are recorded on a detector (Figure 3). The diffraction pattern is then used to work out the structure of the protein. However, whereas x-rays are scattered by the electrons surrounding the nucleus of an atom, neutrons are scattered by the nucleus itself through a nuclear force called the strong interaction. The nucleus of a hydrogen atom, and in particular that of its deuterium isotope, interacts with neutrons very efficiently. Neutron crystallography is therefore a powerful probe for locating hydrogen atoms in enzymes, especially if the hydrogen atoms have been replaced by deuterium atoms.

Progress in applying neutron crystallography to studying enzymes has been slow because it is difficult to produce neutrons in large numbers. Neutron beams are relatively weak compared with x-ray beams, and therefore large crystal sizes are required to scatter enough neutrons to record a clear diffraction pattern. Crystals for studies at x-ray sources are typically a fraction of a millimeter in size, and it can be difficult to grow them larger. When Los Alamos scientist Benno Schoenborn performed the first neutron study of a protein back in 1968 at a nuclear reactor run by Brookhaven National Laboratory, he had to spend several months collecting data from a crystal that was about half the size of a dime. In the years following this pioneering experiment, a number of technical advances at nuclear reactors in the United States, Japan, and Europe provided more facilities for neutron protein crystallography and pushed the required crystal sizes down below 1 millimeter and the time needed to collect neutron diffraction data down to a few days. However, there is little prospect of any further large increase in the flux of reactor neutron sources because of inherent limitations in the nuclear process called fission that is responsible for the production of neutrons.

At another type of neutron source that Los Alamos National Laboratory has been at the forefront of developing, called a spallation source, there are fewer flux limitations. Spallation sources, like the one run by Los Alamos Neutron Science Center (LANSCE), produce neutrons by bombarding a metal target with pulses of high-energy subatomic particles, called protons. Showers of neutrons with a range of energies come off the target in bursts 20 times a second. At the target, the duration of each pulse or burst of neutrons is very short, but as the neutrons travel down beam lines, the pulses become longer because neutrons with different energies travel at different speeds (Figure 4). The low-energy neutrons cannnot keep up with the high-energy neutrons in the same pulse. As a result, neutrons of different energies are detected in a diffraction pattern at different times. Being able to record diffraction patterns that change in time is a big advantage for neutron crystallography. Although the current spallation neutron source run by LANSCE produces fewer neutrons than a large nuclear reactor, the time structure of the neutron beam allows the neutrons to be used more efficiently and with much lower noise levels. There is also the possibility of upgrading the spallation source in the future to produce much higher neutron fluxes.

Figure 5. The Los Alamos Protein Crystallography Station
The Los Alamos PCS is the only resource of its kind in North America and the first to be built at a spallation neutron source. Data collection from small (millimeter-sized) crystals is possible at the PCS because of the use of time-of-flight methods, together with a number of technological innovations, including a large cylindrical neutron detector and a special neutron moderator that, in effect, increases the neutron flux on the sample.

The New Protein Crystallography Station (PCS)

In order to demonstrate the advantages and potential of spallation neutron sources for protein crystallography, Los Alamos scientists Benno Schoenborn and the author have designed and built a PCS at LANSCE (Figure 5). By carefully tailoring the incoming neutron pulses and optimizing detection of those that are diffracted, a state-of-the art user facility has been created for the national and international structural biology community. Since its commissioning in 2002, the PCS has remained the only resource for neutron crystallography studies of enzymes in North America and the first in the world to be built at a spallation neutron source. The PCS project is funded by the office of Biological and Environmental Research of the U.S. Department of Energy. Up to twenty scientific teams visit Los Alamos every year to use the PCS. Although the PCS has been in operation for only two years, those teams, mostly from universities in the United States, have broken new ground by looking at a number of enzymes never studied before with neutrons. And for some of these, they have been able to pin down specific information on important enzymatic reactions and on how drugs bind to enzymes. The two examples that follow are illustrative of these developments.

Solving the Mechanism of Xylose Isomerase

D-xylose isomerase is an enzyme that a number of bacteria use to convert one type of sugar to another (D-xylose to D-xylulose) through a process called hydrogen atom transfer. Not only is this enzyme responsible for a crucial step in the sugar metabolic pathway, and therefore the growth, of a number of bacteria, but it is also used in the food industry to catalyze the conversion of glucose to fructose. A mixture of glucose and fructose, in the form of high-fructose corn syrup, is a high-powered sweetening agent used as a sugar replacement, and D-xylose isomerase has become one of the largest volume commercial enzymes of today. One of the first experiments on the PCS was carried out by a team of visiting researchers from the Fox Chase Cancer Research Center and the University of Tennessee, which is paving the way for scientists to learn exactly how D-xylose isomerase works.

Through previous x-ray diffraction studies, researchers know how the heavier atoms—carbon, oxygen, and nitrogen—make up the molecular structure. The enzyme looks like a barrel or cup with a long handle attached (Figure 6). The walls of the barrel are made from panels of amino acid chains that are folded into sheets on the inside and helices on the outside. At the bottom of the barrel is the active site where the sugary transformation takes place. We know that two metal atoms, usually magnesium atoms, are present in the active site and are important for binding the sugar substrate and also for catalysis. The chief mystery with D-xylose isomerase is where the hydrogen atoms are located in the active site and how they are shuffled during binding and catalysis.

From an industrial point of view, understanding the details of how D-xylose isomerase works could provide clues on how reaction rates could be made faster and higher fructose concentrations achieved, as well as on improving other areas of the production process. However, as team leader Gerry Bunick puts it, “it’s not simply to create a more corn-syrupy world: Better understanding of that particular hydrogen-atom transfer process could lead to improved and better targeted medicines and advances in nanotech-

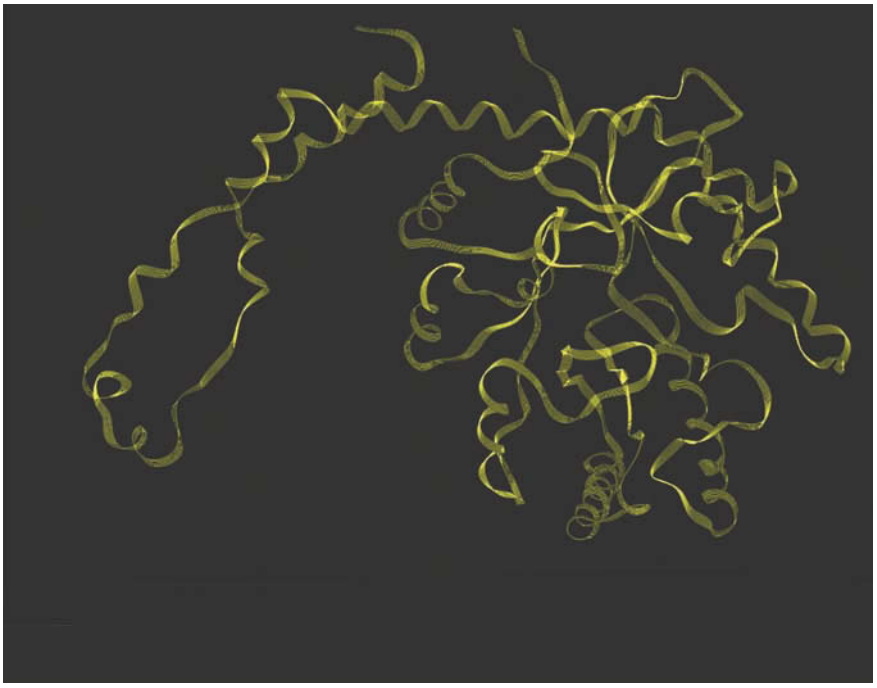


Figure 6. Structure of D-xylose Isomerase
Xylose isomerase, an enzyme responsible for the crucial first step in the metabolism of sugar by bacteria, looks like a cup with a long handle. The active site for the metabolic reactions is deep inside, at the bottom of the cup.

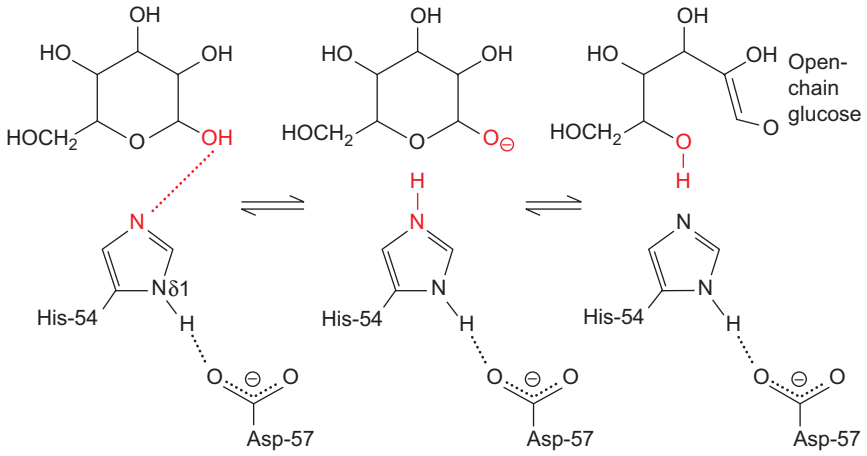


Figure 7. Assumed Initial Steps of Sugar Metabolism by Xylose Isomerase
To break apart the ring of cyclic glucose, D-xylose isomerase was thought to catalyze the transfer of a hydrogen atom from one oxygen in the ring to another. This hypothesis could not be confirmed with x-ray crystallography because none of the details concerning the hydrogen-bonding arrangement of water molecules or the presence of water ions are visible to x-rays.

nology." In particular, the Fox Chase members of the team are interested in the roles of magnesium and water in the function of the enzyme—knowing where the hydrogens are located is a key to understanding those processes. That knowledge will be useful in working with magnesium-containing enzymes that are important to cancer research.

X-ray crystallography has suggested a general pathway for the chemical reaction that involves a number of distinct steps. There are two steps where hydrogen transfer is involved in the catalytic mechanism. One involves opening the ring of the cyclic sugar into a straight chain that the enzyme can then isomerize. The second step involves the transfer of a hydrogen atom between neighboring carbon atoms on the extended sugar chain.

A key histidine amino acid residue in the D-xylose isomerase active site, labeled His54 in Figures 7 and 8, is thought to play a role in opening the sugar ring. The important part of this residue is a flat ring made up from three carbon and two nitrogen atoms. Histidines are unusual because they can have a number of different possible electronic states, depending on whether the nitrogen atoms, labeled $N_{\delta 1}$ and $N_{\varepsilon 2}$, have an attached hydrogen atom or not. Before sugar binds to D-xylose isomerase, the active site is filled with a network of ordered water molecules. One of those water molecules hydrogen bonds to $N_{\varepsilon 2}$ of His54. A water molecule has two hydrogen atoms and an oxygen atom, and knowing whether one of the hydrogen atoms or the oxygen atom is bonding to His54 is important. If $N_{\varepsilon 2}$ does not have an attached hydrogen atom, then it must bond to a hydrogen atom of a water molecule. Therefore, when the sugar substrate displaces water molecules in order to bind in the active site, $N_{\varepsilon 2}$ must bond to a sugar hydrogen atom (Figure 7). Alternatively, if $N_{\varepsilon 2}$ does have an attached hydrogen atom, then it must bond to the oxygen atom of a water molecule, or when sugar is bound, to the oxygen atom of the sugar ring (Figure 8).

For the first experiment on the PCS, crystals of D-xylose isomerase

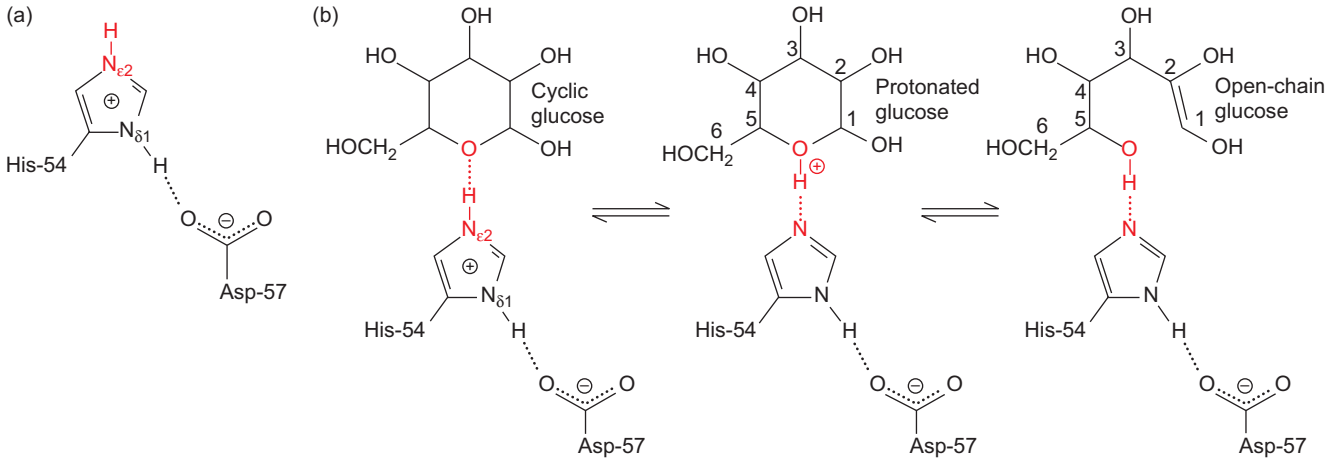


Figure 8. Neutron Results for Breaking the Ring in Cyclic Glucose
(a) The hydrogen atom on $N_{\varepsilon 2}$ in the His54 residue in D-xylose isomerase is deduced from neutron crystallography at the PCS. (b) The hydrogen on $N_{\varepsilon 2}$ first forms a bond with the oxygen on the sugar ring, then it is transferred to that oxygen because His54 is configured to act like an acid, and finally, by that transfer, it causes the sugar ring to break. This mechanism, revealed by neutron diffraction, should be compared with the one shown in Figure 7.

were prepared with no sugar present. The neutron diffraction patterns that were recorded have allowed the visiting researchers to pinpoint where the hydrogen atoms are in the active site. $N_{\varepsilon 2}$ is found to have an attached hydrogen atom that bonds to the oxygen of a water molecule, and what is more, $N_{\delta 1}$ is also found to have an attached hydrogen atom and to be hydrogen-bonded to one of the other amino acid residues (Figure 8). This type of arrangement leads to the hydrogen atom on $N_{\varepsilon 2}$ being very reactive, and it can be easily given up to another molecule in a chemical reaction. The importance of this finding is that it immediately reveals the mechanism for breaking the sugar ring. As shown in Figure 8, the oxygen atom in the sugar ring forms a bond with the hydrogen on $N_{\varepsilon 2}$. However, the His54 is configured to act as an acid and to transfer the hydrogen atom on $N_{\varepsilon 2}$ to the sugar ring oxygen atom, an event that breaks the sugar ring open. A number of other important observations have been made that will be crucial in clearing up details of subsequent reaction steps.

The next experiments on the PCS, which are already underway, involve preparing crystals of D-xylose isomerase with the sugar bound in the active site in order to unravel the details of the second step, in which hydrogen atom transfer is involved in enzyme mechanism. What has been achieved so far is a breakthrough not only from a scientific point of view, but also from a technical point of view. At the time of this experiment, D-xylose isomerase was the largest enzyme ever to have been studied to high resolution using neutron crystallography, an achievement that made the cover of the Winter 2002 issue of the *American Crystallographic Association Newsletter* and also the "Search and Discovery" section of the journal *Physics Today*. As Bunick puts it, "In the case of light elements such as hydrogen, x-rays diffract very weakly. What we were able to do with neutron beams in comparison exceeded everyone's expectations."

DHFR and Antitumor Therapy

Yet another experimental team from the University of Tennessee, this time led by Chris Dealwis, has been studying a similarly large enzyme, dihydrofolate reductase (DHFR). This enzyme binds a small molecule called dihydrofolate and then helps to convert it to tetrahydrofolate, commonly called folic acid, a B vitamin. Before DHFR can shuffle hydrogen atoms about on folic acid, it must bind a helper or cofactor molecule called NADPH. Many metabolic pathways in the cell, in particular those involved in generating the components of proteins and the genetic material DNA, amino acids, and nucleotides, require DHFR along with its cofactor as a specialist enzyme. Blocking the activity of DHFR interrupts the cell's ability to produce DNA and ultimately leads to cell death. X-ray diffraction has shown that the enzyme has two domains with a large cleft where the domains join. NADPH and dihydrofolate have shapes that fit snuggly into different pockets in the cleft.

Several drugs have been discovered that block the action of DHFR, and this effect has been of great use in antitumor therapy. On its first visit to Los Alamos last year, the experimental team collected neutron data from DHFR with the anticancer drug meth-

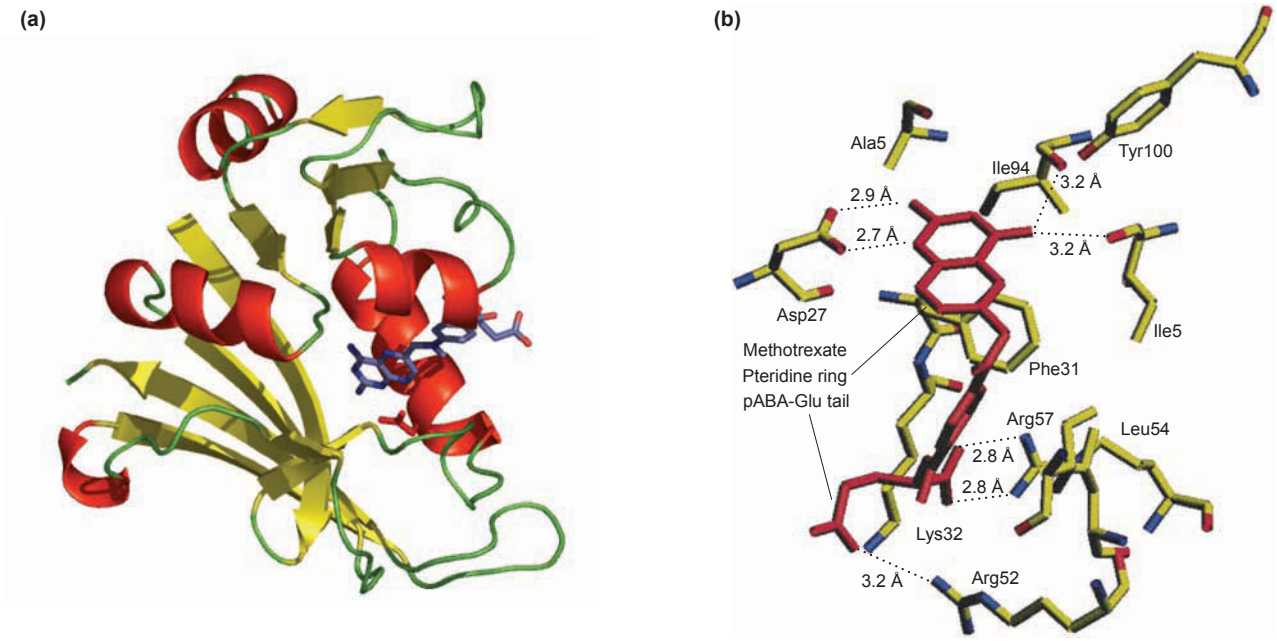


Figure 9. Dihydrofolate Reductase with Methotrexate Bound
(a) This diagram shows the structure of DHFR with the anticancer drug methotrexate bound to the active site. This structure was determined using neutron diffraction. (b) A detail of the binding of methotrexate to the active site is shown. Neutron data are revealing the hydrogen bonds that produce the tight binding of these two.

otrexate bound (Figure 9). Information on where the hydrogen atoms are around methotrexate when it is bound to the enzyme will provide insight that may be key in guiding future anticancer drug design. Unfortunately, the initial diffraction patterns were too weak to allow hydrogen atoms to be visualized. At about 250 times smaller than an inch, the crystals, although large enough for synchrotron x-rays, were too small for neutrons. One of the team members, Brad Bennett, investigated two approaches to overcoming this problem: trying different ways to grow larger crystals and replacing all the hydrogen atoms in DHFR by deuterium atoms, a process called deuteration.

Deuteration makes crystals scatter neutrons up to 10 times more strongly and reduces background noise because deuterium scatters neutrons more efficiently than hydrogen. A biological deuteration laboratory is now in operation at LANSCE churning out deuterate proteins for the PCS user program. DHFR was the first protein to be produced at this facility in a pilot project carried out by Bennett in April 2004. Bennett's efforts in crystal growth and deuteration allowed strong neutron-diffraction patterns to be collected in March 2005, and these data are now providing crucial information about why methotrexate binds so strongly to the enzyme. In fact, by directly visualizing individual protons in the binding site the researchers have found one particular hydrogen atom that they think is key to the binding strength of the drug, an important result for future drug design, which is currently being published in the scientific literature.

The experiments on D-xylose isomerase and dihydrofolate reductase are just two examples from a number of enzymes that have been studied on the PCS so far. Although an increasing number of enzymes are now being studied by neutron protein crystallography, this emerging technique in itself will never be sufficient to determine an enzyme's mechanism or to get a drug to market. It is much more efficient to determine the skeleton of an enzyme structure using synchrotron x-ray or nuclear-magnetic-resonance techniques. However, only neutron protein crystallography can provide the complementary information about how hydrogen atoms are shuffled about this skeleton during enzyme reactions. Structure-based drug design is not one but a collection of technologies that include molecular biology, computational chemistry, bioinformatics, and structural genomics. Los Alamos has provided the nation with a new and unique capability, spallation neutron protein crystallography, that is developing into another, complementary technology in structure-based drug design. However, the role of the PCS goes beyond drug design to providing information on the biochemical steps that underpin our overall understanding of cellular processes and of how defective and pathogenic enzymes affect our health. ■